Stability of RNA from the Retina and Retinal Pigment Epithelium in a Porcine Model Simulating Human Eye Bank Conditions

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Purpose. To assess RNA stability after death in a porcine model to simulate current human eye bank techniques.

Methods. Eye bank time interval data were collected from 191 donor specimens: death to refrigeration, enucleation, and tissue processing. A control porcine eye was enucleated, retina and RPE isolated, and specimens frozen (−80°C). Fourteen porcine eyes remained at room temperature for 2 hours and then cooled to 4°C. Retina and RPE were isolated and frozen (−80°C) at 5, 12, 24, 29, 36, 48, and 72 hours. Four globes remained in a moist chamber, five whole and five sectioned globes were immersed in RNA*later* (Ambion, Austin, TX) at 5, 12, 24, or 48 hours. RNA was isolated. The 28S and 18S rRNA peaks were analyzed by electrophoresis. RT-PCR was performed on each sample. Messenger RNA for GAPDH, β-actin, mouse rhodopsin from retina (mRHO), and RPE-65 (from RPE) were analyzed with gel electrophoresis.

Results. The average time from death to refrigeration was 4.2 hours, to enucleation 6.4 hours, and to tissue processing 10.7 hours. RT-PCR gel electrophoresis patterns from retinal tissue had bands of similar intensity at each interval from β-actin, GAPDH, and RHO. Band patterns from RPE demonstrated decay of the RT-PCR gene products after 5 hours. This decay was delayed by at least 24 hours with the use of RNA*later*. The 28S rRNA decay was similar for retina and RPE.

Conclusions. Retinal tissue RNA can be analyzed within the time constraints of current eye bank tissue processing, whereas analysis of RPE necessitates either rapid processing or use of RNA*later*. These results should aid in future studies in which eye bank tissue is used for RNA analysis. (*Invest Ophthalmol Vis Sci.* 2003;44:2730–2735) DOI:10.1167/iovs.02-1120

Identification of mRNA from tissue samples may provide valuable molecular clues to study a variety of ocular diseases. Stability of these labile molecules is highly variable. At the time of death, an RNA profile is present within various ocular tissues that may be correlated to an identifiable ocular disease process. Immediately after death, the balance of RNA production and degradation is altered, and many variables affect this balance. Vision researchers are fortunate to benefit from human eye donors. Eye banks provide a valuable source of human tissue for corneal transplantation, and they also provide valuable tissue for studying human ocular disease. In the current study, we established average eye bank tissue processing times and simulated eye bank techniques in a porcine model to determine the stability of various RNA molecules from the retina and retinal pigment epithelium (RPE) over time. The goal of this study was to identify the postmortem change in the balance of RNA degradation in the retina and RPE, in a porcine model that simulated current eye bank techniques. Knowledge of RNA degradation patterns in the retina and RPE will establish a more accurate template for further study of the molecular pathogenesis of ocular disease using human eye bank tissue.

Methods

Eye Bank

The Minnesota Lions Eye Bank collected data from regional sites (multistate area) as well as from the Minneapolis and St. Paul metropolitan area for 191 consecutive specimens (Table 1). Time of death to refrigeration of the cadaver, enucleation of the eye, and preservation of tissue was recorded and averaged for various regions.

Porcine Eye Conditions

Porcine eyes were collected from unrelated experiments conducted at the University of Minnesota. Pigs were not killed specifically for this experiment. Either whole globes or half globes (posterior segments) were used. The first eye was enucleated immediately after death, dissected, snap frozen in liquid nitrogen, and stored at −80°C. The first 10 eyes were whole globes. Eyes 11 to 15 were transected to simulate the removal of the anterior segment in the eye bank. Posterior segments were then subject to various conditions. Eyes 2 to 15 were enucleated and placed in a moist chamber at room temperature for 2 hours and then cooled to 4°C. Globes 6 to 15 were suspended in 30 mL of RNA*later* (Ambion, Austin, TX) at various time intervals and remained suspended for 24 hours before specimens were dissected and frozen at various time points (Fig. 1).

Retina and RPE Isolation

Globes were transected anterior to the equator with a razor blade. Dissection was slightly different for globes placed in RNA*later* versus those in the moist chamber. Normal saline was used to detach the retina in globes maintained in the moist chamber. The retina was excised at the optic nerve and placed in a tube (Eppendorf, Fremont, CA). RPE cells were gently collected with a small paintbrush and transferred to a tube (Eppendorf). Eyes treated with RNA*later* demonstrated adherence between the retina and the RPE. To separate these tissues, the retina and RPE were placed in a tube (Eppendorf) filled with 0.5 mL normal saline and gently agitated 15 to 20 times to separate the RPE. The retina was removed, placed a separate tube with 0.3 mL normal saline, and reagitated. This process was repeated a third time to release the RPE. All tubes were RNase free. The retina and RPE specimens were immediately frozen in liquid nitrogen and then transferred to −80°C for storage.

Isolation of RNA

Frozen tubes of retina and RPE were allowed to thaw for 5 minutes at room temperature. Tubes were centrifuged for 5 minutes at 3000*g*.

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TABLE 1. Minnesota Eye Bank Data from 191 Human Eye Donors, with Average times from Death to Refrigeration, Enucleation, and Globe Processing

	Time to Refrigeration	Time to Enucleation	Time to Processing
Minimum	2.2	4.9	8.3
Maximum	5.9	9.9	12.9
Metropolitan Area	3.5	5.4	8.3
Mean	4.2	6.4	10.7

The metropolitan area includes sources from within the Twin Cities of Minneapolis and St. Paul, Minnesota. All regional sites for the Minnesota Lions Eye Bank are included in the minimum and maximum. The mean includes all sites. Times are in hours.

RNA from both retinal and RPE specimens was isolated with extraction reagent (TRIzol reagent; Invitrogen-Life Technologies, Carlsbad, CA, and RNeasy Mini Kit with On-column DNase Digestion; Qiagen, Valencia, CA). All tissues were homogenized with a motorized tube pestle (Eppendorf) suspended in 1 mL of the extraction reagent. After a 5-minute incubation, 0.2 mL chloroform was added and the tube was shaken vigorously, allowed to sit at room temperature for 5 minutes, and centrifuged at 11,000*g* for 15 minutes. The aqueous phase was collected, and RNA was precipitated with 0.5 mL isopropyl alcohol and centrifuged at 11,000*g* for 10 minutes. The pellet was washed with 1 mL of 80% ethanol, and centrifuged at 7500*g* for 5 minutes. The ethanol was removed with a pipette, and the pellet was allowed to air dry for 15 minutes. The RNA was then redissolved in 50 μL of RNase-free water. RNA was purified further with a kit (RNeasy Mini Kit; Qiagen). Yields of RNA were determined spectrophotometrically.

RNA Microchip Electrophoresis

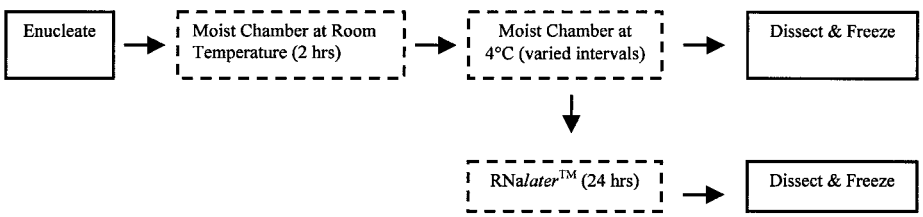
RNA analysis was performed on a bioanalyzer (model 2100; Agilent Technologies, Palo Alto, CA, using the RNA 6000 LabChips kit). All chips were prepared and loaded with 1 mL of RNA samples of both retina and RPE from eyes 1 to 5. Analysis was performed on computer (Biosizing software; Agilent).

Reverse Transcriptase–Polymerase Chain Reaction

A commercial reverse transcriptase protocol (OmniScript; Qiagen) was performed with 4 μg of total RNA from each sample. PCR reactions using a duplex primer set for β-actin and a triple primer set for GAPDH were performed (Table 2). PCR reactions were performed on 2 μL of each 20-μL RT sample, using *Taq* DNA-polymerase (Invitrogen-Life Technologies). The following PCR settings were used: 94°C for 5 minutes followed by 30 repeating cycles of 94°C (30 seconds), 55°C (30 seconds), and 72°C (60 seconds). The PCR product was analyzed by 2% agarose gel electrophoresis.

Tissue-Specific Genes

Primers from retina- and RPE-specific genes were used for PCR. PCR conditions for tissue-specific genes were performed as described. The PCR product was isolated, and the sequence was confirmed with both 5′ primer and 3′ primer. The retina-specific gene was from the mouse rhodopsin gene sequence (mRHO primers 5′-CCAGCAGCAGGAGTCAGCCACC-3′, 5′-GGCTGGTCTCCGTCTTGGAAGCGG-3′), and the RPE-specific gene was from mouse RPE-65 (primers 5′-AATGGATTTCTGATTGTGGA-3′, 5′-TCAGGATCTTTTGAACAGTC-3′).



TIME (hrs)		0	2	5	12	24	29	36	48	72
Whole Globes	1	E → D → F								
	2	E → M	C	D → F						
	3	E → M	C		D → F					
	4	E → M	C			D → F				
	5	E → M	C				D → F			
Whole Globes with RNAlater	6	E → R	C			D → F				
	7	E → M	C	R			D → F			
	8	E → M	C		R			D → F		
	9	E → M	C			R			D → F	
	10	E → M	C				R			D → F
Posterior Globes with RNAlater	11	E → R	C			D → F				
	12	E → M	C	R			D → F			
	13	E → M	C		R			D → F		
	14	E → M	C			R			D → F	
	15	E → M	C				R			D → F

FIGURE 1. Fifteen globes were used in the experiment: whole globes (globes 1–5), whole globes with RNA*later* (Ambion, Fort Worth, TX; globes 6–10), and posterior globes with RNA*later* (globes 11–15). Time intervals after enucleation (E), placement in moist chambers (M), refrigeration (C), RNA*later* use (R), dissection (D), and freezing (F) are indicated.

TABLE 2. β-Actin and GAPDH Primers Used for Polymerase Chain Reactions with Porcine cDNA

Duplex Primer Set	
β-Actin outer sense (bp 402–425)	5′ CAGATCATGTTTGAGACCTTCAAC 3′
β-Actin inner sense (bp 861–883)	5′ ATCCACGAAACTACCTTCAACTC 3′
β-Actin anti-sense (bp 1016–1038)	5′ GAGGAGCAATGATCTTGATCTTC 3′
Triple Primer Set	
GAPDH outer sense (bp 528–547)	5′ GGGAAGCTTGTCATCAATGG 3′
GAPDH middle sense (bp 820–839)	5′ CCAAGGTCATCCATGACAAC 3′
GAPDH inner sense (bp 1150–1169)	5′ GCATCCTGGGCTACACTGAG 3′
GAPDH anti-sense (bp 1344–1364)	5′ CTTTACTCCTTGGAGGCCATG 3′

Primers were chosen that were identical for both humans and pigs as determined by the NCBI Blast search (http://www.ncbi.nlm.nih.gov).

RESULTS

The average postmortem events that occur in current eye banking are summarized in Table 1. The time intervals represent averages from various geographic locations in and around the Twin Cities (Minneapolis and St. Paul). We attempted to simulate postmortem temperature changes, an important variable, in our porcine model. The minimum average time that the body remains at room temperature after being pronounced dead is approximately 2 hours. Tissue from the Minnesota Lions Eye Bank comes primarily from the metropolitan area (78/191 or 41%). The average time from death to refrigeration (4°C) in the metropolitan region was 3.5 hours, whereas the best location average was approximately 2 hours. Average enucleation times in various locations ranged from 4.9 to 9.9 hours after death (metropolitan average was 5.4 hours). Whole globes are immediately placed in a moist chamber in an insulated container on ice (4°C) and transferred to the eye bank for tissue processing (metropolitan area average was 8.3 hours). Some tissue from regions farther away had higher average processing times (maximum was 12.9 hours). Globes are then immediately processed at the eye bank and examined for potential use in corneal transplantation. Anterior segments are removed, and posterior globes are then available for research purposes. If the cornea is not useful for transplantation, whole globes may be available for research, and in some circumstances, tissue may be designated for research at the time of enucleation.

Porcine eyes were treated in a manner that closely simulates the ideal eye bank conditions (Figure 1). Based on our data, the minimum average time for human eyes from death to refrigeration was approximately 2 hours and minimum average enucleation time was approximately 5 hours (Table 1). Globes from the eye bank are available, either as whole globes or posterior segments. To assess the tissue effects of RNA*later*, both whole globes and posterior segments of porcine eyes were immersed in RNA*later* and maintained at 4°C (Table 1). Whole globes placed in RNA*later* appeared dehydrated and shrunken. Posterior segments in RNA*later* were similar in appearance to those placed only in moist chamber vials.

Total RNA from retina and RPE samples were isolated by using a combination of guanidinium isothiocyanate (GITC) extraction and silica-based filter-binding methods. We used extraction reagent (TRIzol; Invitrogen-Life Technologies) to isolate crude total RNA and removed DNA and protein contamination with separation columns (RNeasy; Qiagen). We found that RNA samples from RPE cells extracted with the reagent alone appeared pigmented. After decontamination (RNeasy; Qiagen), samples were then pigment free. Total RNA yield from the retina of a single porcine eye ranging from 32 to 67 μg as assessed spectrophotometrically, whereas from RPE, the yield was much lower at approximately 8 to 39 μg/eye. After separation column clean up (RNeasy; Qiagen), OD_{260} was reduced by 40% to 60%, due to DNA contamination.

Total RNA from each sample was used for RT-PCR. In addition to the β-actin primers used by Sugita et al.,[1] we designed the one-step multiple primer set for GAPDH to assess mRNA integrity (Fig. 2). The RT-PCR products from β-actin primers form two bands, long (637 bp) and short (178 bp), whereas the RT-PCR products from GAPDH gels produce three bands: long (817 bp), medium (525 bp), and short (195 bp). The presence of the long band in the RT-PCR product indicates less degraded RNA.

RT-PCR of RPE

Both β-actin and GAPDH PCR amplification products (two bands for β-actin and three bands for GAPDH) were present in

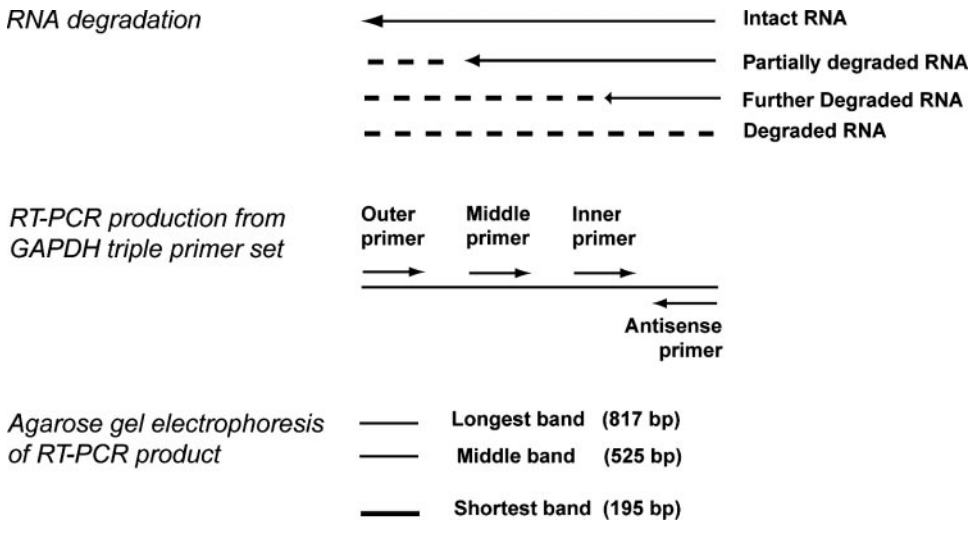


FIGURE 2. The triple primer set. mRNA degraded with time and produced smaller mRNA segments. RT-PCR was conducted with three primers and an equal amount of antisense primer. Agarose-gel electrophoresis of the combined PCR product demonstrated various bands that correspond with the RT-PCR gene product (see Fig. 4). The schematic is modeled after a depiction by Sugita et al.[1]

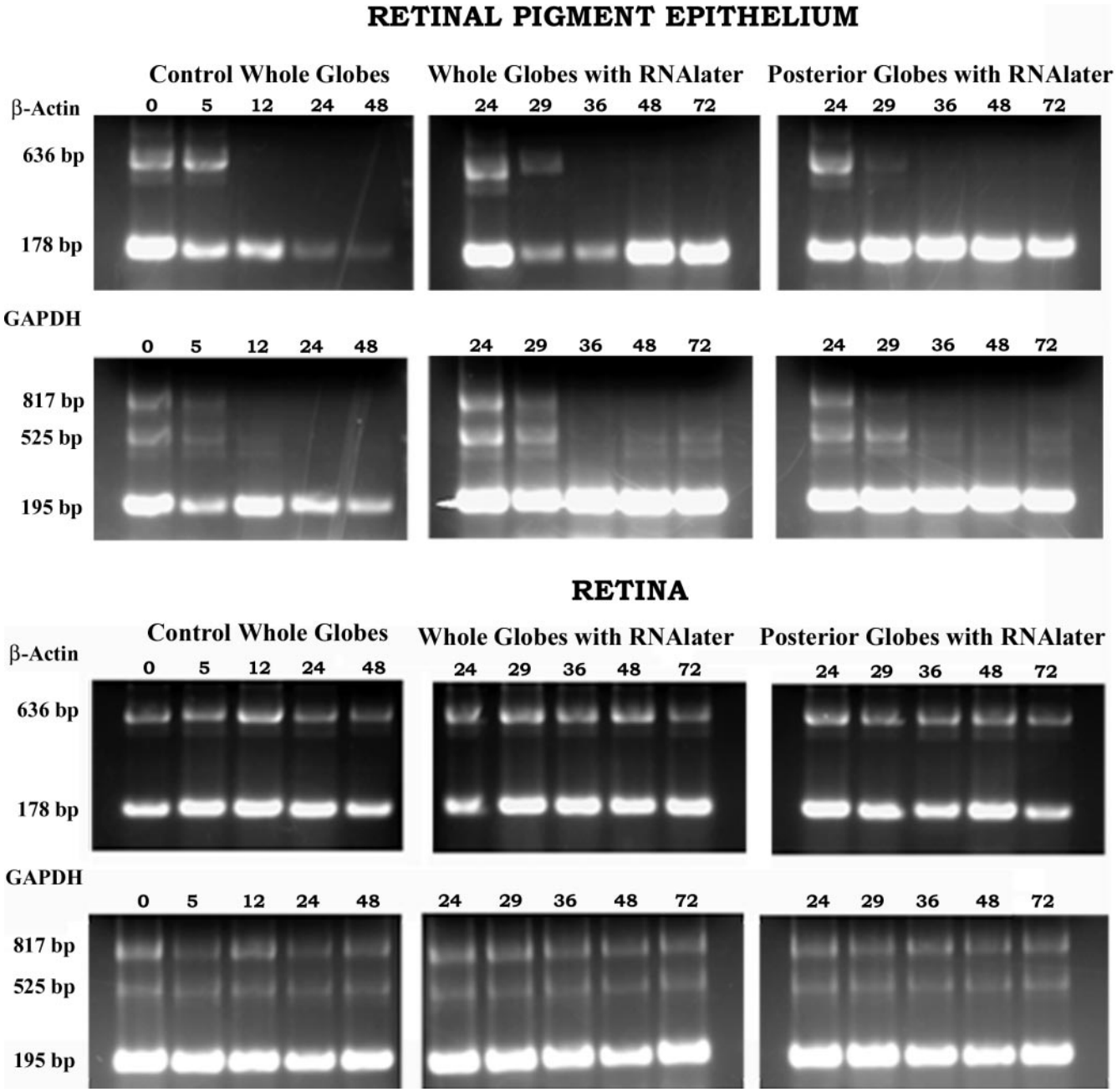


FIGURE 3. Gel electrophoresis pattern of RT-PCR gene products from RPE (*top*) and retina (*bottom*) at various time intervals, with either GAPDH or β-actin. After 5 hours there was an observable loss of the longer bands for the RPE RT-PCR gene products. RNA*later*, however, preserved these gene products for approximately 24 hours. Finally, RT-PCR gene products for retinal tissue was relatively stable at all time intervals.

the control eye (globe 1; Fig. 3, top). After 5 hours of previously specified conditions, both β-actin and GAPDH PCR bands are present. The short (178 bp) β-actin PCR band at and beyond 12 hours is still present, whereas the long (636 bp) β-actin PCR band is not present. The short (195 bp) GAPDH PCR band at and beyond 12 hours is still present. The medium (525 bp) and long (817 bp) GAPDH PCR products were greatly diminished. The mRNA from RPE cells appeared to degrade approximately 5 to 12 hours after death.

RT-PCR of RPE in RNA*later*

Whole globes or posterior chambers preserved in RNA*later* for 24 hours after enucleation had PCR band intensity similar to that of the control for both β-actin and GAPDH (Fig. 3, top). All samples preserved in RNA*later* showed RT-PCR pattern similar to that of the whole-globe control, in which dissections were performed immediately at the predetermined time interval.

RT-PCR of Retina

Both β-actin and GAPDH mRNA demonstrated stability over 48 hours in control eyes, as demonstrated by the presence of all bands (Fig. 3, bottom). Again, treatment of whole globes or posterior segments with RNA*later* did not alter the band intensity at any time interval.

RT-PCRs for Tissue-Specific Genes

To determine the relevance of studying housekeeping genes compared with tissue-specific genes for the retina and RPE in control globes, we also generated data from PCR of tissue-

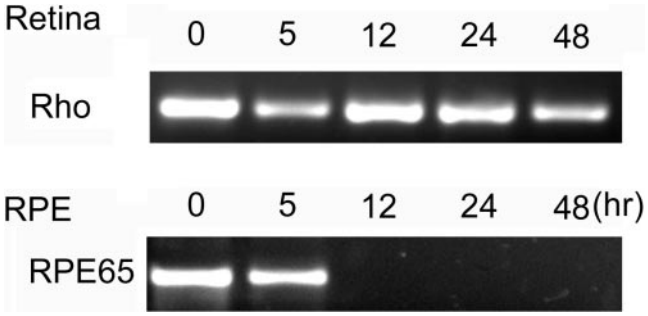


FIGURE 4. Gel electrophoresis pattern of RT-PCR gene products from tissue-specific genes of retina (RHO, *top*) and RPE (RPE-65, *bottom*). Note that the retinal gene product was present at each interval, whereas there was a loss of the RPE gene product after 5 hours.

specific genes RHO and RPE-65 (Fig. 4). Once again, RPE-65 PCR products were present only at time 0 and 5 hours, whereas the retinal tissue mRNA was relatively stable at all time points tested. These results demonstrated mRNA degradation patterns nearly identical with those of the housekeeping genes β-actin and GAPDH.

Ribosomal RNA 18S and 28S

To confirm the RT-PCR results, the quality of RNA from retina and RPE, samples taken from the control group were further analyzed with microchip electrophoresis (Bioanalyzer; Agilent). Retinal RNA samples demonstrated clear 28S and 18S rRNA peaks through 24 hours, whereas samples from 48 hours showed degradation of the 28S peak with lower amplitude and smaller peaks (Fig. 5). However, RPE samples demonstrated a more profound reduction of the 28S peak after 5 hours that continued to decay with time.

DISCUSSION

Ocular diseases may be studied by examining the gene expression profile from available tissue. Eye banks are an excellent source of ophthalmic tissue evaluation that can also be examined clinically for phenotypic changes of ocular disease. DNA microarray[2–4] and serial analysis of gene expression (SAGE)[5] represent powerful new methodologies to examine global gene expression and to identify candidate genes. Both techniques rely on mRNA quality to represent the gene expression profile at the time of death. In our study we examined the porcine eye using a "best possible scenario" approximation of current eye bank techniques with corresponding specific time intervals, carefully controlled in a pig model, to simulate the quality and stability of mRNA from tissue samples at various postmortem time intervals. The implications of our data establish a useful time frame and offer valuable quality control measures for assessing the quality of available tissue for gene expression studies. Translation of the pig model to human tissue has certain limitations. Necessary controls used in this study would not be possible when using human tissue. However, the techniques are certainly applicable to human eye bank tissue.

Cellular mRNA transcripts have highly variable rates of decay, with some lasting only minutes.[6] Eye bank techniques with postmortem variables (time until refrigeration, enucleation, and preservation) have an unknown effect on mRNA stability. Wang et al.[7] have shown in human ocular trabecular meshwork tissue that FOXC1 mRNA degrades rapidly after death. Furthermore, they demonstrated delayed degradation of the FOXC1 mRNA with the addition of RNA*later* compared with samples stored on ice or frozen at −80°C. They report an average postmortem interval (death to tissue processing) of less than 5 hours. They preserved the tissue in a moist chamber, by rapid freezing, by rapid freezing of whole-globes on dry ice, or by placing whole globes in RNA*later*. During tissue processing with RNA*later*, whole globes were cut, the vitreous removed, and specimens submerged in RNA*later*.

In the present study, we used the porcine eye and established an ideal postmortem control by immediate postmortem enucleation, dissection, and rapid freezing. This sample provides the best approximation of mRNA quality that can be used for comparing specified postmortem time intervals using various methods of tissue preservation. Specific processing times were chosen based on "best-case scenario" eye-banking intervals (Table 1). Finally, we demonstrated that placing the tissue directly into RNA*later* solution similarly preserved either whole globes or sectioned globes with equivalent efficacy, even without manipulation of the vitreous.

The mechanism of RNA decay begins with deadenylation of the 3′ poly-A tail, decapping of the 5′ end, and degradation by the 5′ and 3′ exonuclease.[6] Degradation by the 5′ exonuclease plays a major role in mRNA decay.[8] The most common procedure to analyze the integrity of RNA is by fractionating total RNA on a denaturing agarose gel. Using this method, the intensity of the 28S rRNA and 18S rRNA bands reflects the degree of RNA degradation.[9,10] Herein, we used the bioanalysis (Agilent) to quantitatively compare 28S and 18S rRNA levels. Either this method or RT-PCR is feasible, especially when only a small amount of RNA is available. Ocular tissues, such as the RPE, necessitate the use of extremely small sample sizes. Use of the traditional method requires that most of the isolated RNA be used in screening, rendering it unavailable for further molecular profiling.

Sugita et al.[1] first discussed the one-step method for the evaluation of mRNA gene stability using multiple primers for PCR of human β-actin, a housekeeping gene. They describe a method to assess mRNA degradation using a density ratio of the two band segments created in the duplex primer set. In our study, we used three primers for PCR of human β-actin and four primers of GAPDH to monitor mRNA degradation. The relative ratio of the PCR product determines the degree of mRNA degradation and offers a more comprehensive and precise assessment of mRNA stability by detecting earlier decay, thus providing a more complete profile of postmortem changes from these two ocular tissues. Finally, use of multiple primer sets could serve as quality control measures for assess-

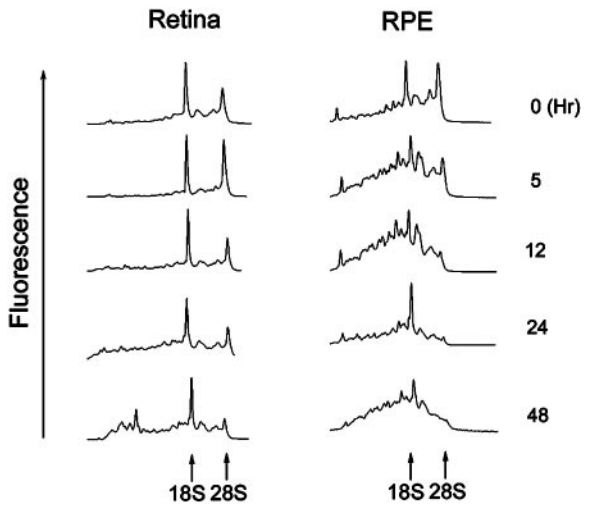


FIGURE 5. Microchip bioanalysis of rRNA 18S and 28S at various time intervals. The 28S peak decayed slowly in retinal tissue at 24 hours, whereas the decay of the 28S peak from RPE tissue declined rapidly after 5 hours.

ment of tissue that is subsequently analyzed with microarray or SAGE.

Retinal tissue mRNA bands of β-actin and GAPDH housekeeping genes as well as the retina-specific rhodopsin gene were present at all time intervals after death with each of our globe-preservation techniques. Perhaps the intracellular environment of retinal tissue offers prolonged protection from postmortem nuclease activity. We cannot determine whether the production of mRNA continues after death, but the balance does not appear to favor production during the time intervals tested. Retinal tissue is composed of multiple cell types, such as photoreceptors, Müller cells, ganglion cells, and other neurons. Housekeeping mRNA (representing all retinal cells), as well as tissue-specific mRNA (photoreceptors), demonstrate stability at all time intervals. However, we did not evaluate other specific retinal cell markers.

Unlike retinal tissue, RPE mRNA bands of β-actin and GAPDH housekeeping genes as well as the RPE-specific RPE-65 gene demonstrate a relatively rapid decay after death. The benefit of using multiple primers is evident by comparing the 5- to 12-hour postmortem band patterns of GAPDH or β-actin (Fig. 3, top). After 5 hours, disappearance of the longer bands (817 bp GAPDH and 636 bp β-actin) demonstrates mRNA instability of both housekeeping genes in RPE. Similarly, the disappearance of the RPE-65 band after 5 hours confirms this critical interval for evaluating mRNA from RPE. The RPE is a highly metabolic tissue responsible for maintaining the visual cycle and processing photoreceptor outer segments. In addition, the RPE is a monolayer of cells that dissociate rapidly with tissue processing techniques (brushing) and may be more susceptible to environmental influences. Nucleases may be released from lysed RPE and result in further degradation. One could speculate that the unique features and metabolic demands of the RPE make it more susceptible to postmortem mRNA degradation.

The use of RNA*later* for RPE clearly demonstrates prolonged preservation of mRNA integrity. We demonstrated that by rapidly immersing ocular tissue in RNA*later*, we delayed mRNA degradation by at least 24 hours. Wang et al.[7] cut the sclera and removed vitreous during tissue processing. We found that globe sectioning was unnecessary before treating with RNA*later*. The advantage of using RNA*later* with eye bank tissue is that personnel can simply place whole globes or posterior segments in RNA*later* as soon as tissue is available or adequately processed to remove corneal tissue. The Minnesota Lions Eye Bank has an average death-until-enucleation time of approximately 5 hours for tissue obtained within the metropolitan area. Eye bank personnel can place selected tissue in RNA*later* as a whole globe immediately after enucleation. Tissue may then be preserved for dissection and freezing the following day. We estimate that the ocular tissue immersed in RNA*later* by 5 hours after death would provide high-quality tissue samples for mRNA analysis. Another observation is that with the use of RNA*later*, ocular tissues seemed to dehydrate. However, the shrunken tissue does not interfere significantly with dissection. Using RNA*later* interferes with protein structure and function; therefore, subsequent protein analysis would be difficult.

In summary, we have established a time-specific model for mRNA degradation using the porcine eye to simulate current eye bank techniques. Our data suggest that, with standard eye bank techniques, mRNA from retinal tissue is well preserved for at least 48 hours after death. However, mRNA from RPE begins to degrade between 5 and 12 hours after death. From the time that RNA*later* is added to the RPE, degradation is delayed by at least 24 hours. Either whole globes or posterior segments may be suspended in RNA*later* to improve mRNA quality and preserve the integrity of the tissue for mRNA analysis. The framework of these mRNA degradation profiles will serve as a useful guide to analyze human tissue for gene expression studies of ocular disease.

Acknowledgments

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